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Pulmonary dendritic cells isolated from neonatal and adult ovine lung tissue

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Abstract

Lung dendritic cells (DCs) are potent antigen presenting cells (APCs) that initiate and modulate the adaptive immune response upon microbial infection within the pulmonary environment. For the first time, neonatal and adult lung DCs in a large animal model were compared in these studies. Here, we isolated and identified lung DCs in both neonatal and adult sheep, a valuable experimental animal utilized in pulmonary studies of naturally occurring respiratory diseases. Neonatal lung DCs exhibited characteristic dendrites and morphology when observed by transmission electron microscopy and expressed low to moderate DEC-205, CD80/86, MHC class II and CD 14. Regardless of age, lung DCs were functionally able to endocytose FITC conjugated ovalbumin but to a lesser degree than monocyte-derived DCs. In addition, neonatal lung DCs were demonstrated to be potent stimulators of allogeneic T cell proliferation. Together, these results demonstrate that neonatal and adult lung DCs are functionally similar. It is apparent from the data presented that neonatal pulmonary DCs do not exhibit an intrinsic functional defect that would impair their ability to take up antigen and stimulate naïve T cells. These data support growing evidence that neonatal immune responses may differ from adults due to different microenvironmental influences rather than differences in dendritic cell maturation states.

Keywords: Ovine lung; Dendritic cells; Neonates

Dendritic cells (DCs) play a vital role in antigen capture, processing, and presentation to T cells. These potent antigen presenting cells (APCs) are a critical link between innate and adaptive immune responses. In response to the appropriate immune or inflammatory signals, DCs migrate from tissues to draining lymph nodes where they present antigen to T cells. DCs are noted for their unique dendritic morphology and ability to stimulate naive T cells that separate DCs from other APCs such as macrophages and B cells. These unique characteristics of DCs contribute to their ability to obtain and present antigen, effectively triggering the adaptive immune response.

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Pulmonary DCs are distributed throughout the lung parenchyma and form a network within the epithelium lining the airways (van Haarst et al., 1994; Schon-Hegrad et al., 1991; Gong et al., 1992; Sertl et al., 1986). Additional lung DCs populations reside within the visceral pleura (Simecka et al., 1986). Rodent models have been used extensively for isolation and identification of lung DCs. The importance of lung DCs has been demonstrated in models with an increase in recruitment of DCs into the lungs after exposure to influenza virus or respiratory syncytial virus (RSV) infection (D'Hulst et al., 2002; Beyer et al., 2004). Steady state and pathogen induced migration of DCs to and from the lung demonstrates the active role these cells have in immune surveillance and protection (Stumbles et al., 2003; McWilliam et al., 1996; Lagranderie et al., 2003).

To date, lung DCs from a large animal species have not been described. The aim of these studies was to compare ovine neonatal and adult lung DCs for phenotypic and functional differences. Monocyte-derived dendritic cells (MDDCs) have been studied and characterized in both bovine and ovine models but no data are available on lung DCs in these species (Chan et al., 2002; Howard et al., 1999; Bajer et al., 2003). The respiratory diseases that occur naturally in the ovine species include RSV and parainfluenza type 3 (Lindgren et al., 1996; Meyerholz et al., 2004; Lehmkuhl and Cutlip, 1983). Utilizing tissue derived dendritic cells from infected lambs will lead to valuable insights for studies on pathogen–host interactions. Here, we developed an isolation procedure to obtain an enriched population of ovine neonatal and adult lung DCs that exhibit characteristic morphology, ultrastructure and phenotype typical of pulmonary DCs isolated from other adult species. In addition, we show that neonatal and adult lung DCs are functionally similar in antigen capture and T cell stimulation. These results are the first reported data for isolated lung DCs in a neonatal and adult large animal model.

1. Materials and methods

1.1. Animals and experimental procedure

Two to four day old lambs ($n = 10$) of mixed Rambouillet and Polypay breeds were used for neonatal lung specimens. Year old sheep ($n = 4$) of the same mixed breeds were used for adult lung specimens. During necropsy, the abdominal cavity was opened and the abdominal aorta severed. Lungs were perfused using a similar procedure to that previously described for rodents (Stumbles et al., 2001). Briefly, the pulmonary artery was clamped as it exits the heart and catheterized with an 18-gauge needle distal to the clamp. The vasculature of the lungs was perfused with sterile PBS until the lungs were cleared of peripheral blood and white in color. The lungs were aseptically removed, lavaged with sterile PBS to remove alveolar macrophages and placed in sterile PBS containing 1% antibiotic-antimycotic (Gibco, Carlsbad, CA). Accepted principles of animal care were followed and experiments approved by the NADC Animal Care and Use Committee.

1.2. Pulmonary DC isolation and enrichment

The entire lung was cut into small pieces using sterile scissors and forceps then digested in RPMI 1640 (Invitrogen, Carlsbad, CA) containing Liberase Blendzyme 3 (7 mg/ml; Roche, Indianapolis, IN) and DNase I, type IV from bovine pancreas (50 μg/ml; Sigma, St. Louis, MO) for 1 h at 37 °C. The enzymatic digestion was stopped by the addition of 10% FBS to the decanted supernatant and the entire digestion step was repeated on remaining tissue pieces. The cell suspension was filtered through sterile cheesecloth and washed with PBS. Contaminating RBCs were removed from cell preparations by hypotonic lysis. Cells were resuspended in supplemented RPMI 1640 (cRPMI) containing 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 10% lamb serum, 2 mM L-glutamine and 1% antibiotic–antimycotic (all from Gibco) and incubated for 12 h at 37 °C in an atmosphere of 5% CO_2. Adherent and nonadherent cells were collected, pooled, washed and incubated for 10 min at room temperature with 10% normal goat serum (Invitrogen, Carlsbad, CA) to block nonspecific binding. Cells were washed and stained with monoclonal antibody BAQ153A (anti-ovine CD 11c; VMRD, Pullman, WA) followed by incubation with magnetic micro beads (Miltenyi Biotech, Auburn, CA). $CD11c^+$ DCs (lung DCs) were obtained by twice positively sorting using a magnetic activated cell sorter (Miltenyi Biotech).

1.3. Lymph node DC isolation

Tracheobronchial and mediastinal lymph nodes were aseptically removed and placed in RPMI 1640. Homogenization of lymph nodes with sterile tissue grinders (Sigma) resulted in single cell suspensions that were incubated in cRPMI for 12 h at 37 °C with 5% CO_2. Isolation of $CD11c^+$ DCs (LN DCs) was similar to that described above for pulmonary DCs.

1.4. Peripheral blood mononuclear cell (PBMC) isolation

Peripheral blood was collected from neonatal lambs and adult sheep into heparinized vacutainer tubes. Tubes were centrifuged at 480 × *g* for 30 min at 4 °C. Buffy coats were harvested and contaminating RBCs removed by lysis. PBMCs were washed and resuspended in either RPMI or cRPMI depending upon the assay.

1.5. Monocyte-derived macrophages and DCs

PBMCs suspended in cRPMI were dispensed into six-well plates (1×10^6 cells/3 ml) and incubated overnight at 37 °C with 5% CO_2. The wells were washed to remove nonadherant cells and cRPMI supplemented with 5 ng/ml of recombinant human GM-CSF and 2 ng/ml of recombinant human IL-4 was added. Medium was changed every 2–3 d and cells were allowed to differentiate into monocyte-derived DCs (MDDCs) for 7 d. Maturation of MDDCs was induced by the addition of *Escherichia coli* LPS O55:B5 (1 μg/ml; Sigma) to culture supernatants for another 72 h. Macrophages were derived from adherent cells that were cultured in cRPMI and harvested on day 7.

1.6. Transmission electron microscopy (TEM)

$CD11c^+$ cells were fixed in 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer. Cells were post-fixed in osmium tetroxide and embedded in Eponate 12 resin. Samples were polymerized at 60 °C for 48 h. Blocks were sectioned and stained with uranyl acetate and Reynolds lead citrate. Samples were viewed using a Tecnai G^2 Biotwin (FEI Company, Hillsboro, OR) transmission electron microscope.

1.7. Immunohistochemistry

Samples of PBS-perfused lung were frozen in OCT medium in an ethanol and dry ice bath. Sections were cut onto silanated glass slides, fixed in acetone for 5 min and stored at −80 °C. Slides were rehydrated in 1× Tris buffer and blocked with normal goat serum (Kirkegaard Perry Labs, Gaithersburg, MD) for 30 min at room temperature. The slides were washed in 1× Tris buffer and incubated with primary antibodies diluted in 1× Tris buffer, PBS and 3% BSA. Primary antibodies included BAQ153A (anti-ovine CD11c) and CC98 (anti-bovine DEC-205), which were incubated at 4 °C overnight. Slides were washed with 1× Tris buffer and incubated for 30 min at 37 °C with isotype specific secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 633 (Molecular Probes). To visualize nuclei, slides were washed and incubated with 4′,6-diamidino-2-phenylindole, dilactate (DAPI, 300 nM; Molecular Probes) for 5 min at room temperature. The slides were washed with 1× Tris buffer and cover slips mounted with a 50–50 glycerin and PBS solution. Tissue sections were examined using a Leica TCS-NT confocal scanning laser microscope (Leica Microsystems, Inc., Exton, PA). Images were prepared using Adobe Photoshop version CS and InDesign version 3.

1.8. Flow cytometry

Isolated $CD11c^+$ cells were analyzed for cell surface antigens using three-color flow cytometry. Cell concentrations were adjusted to 1×10^5 cells/ml and stained using different combinations of primary and secondary antibodies (Abs) diluted in FACs buffer (balanced salt solution with 1% FBS and 0.1% sodium azide). Ovine specific monoclonal Abs obtained from VMRD were used for detection of CD14 (CAM36A), CD11c (BAQ153A), MHC class I (BC5), and MHC class II (TH14B). Cross-reactive CD11b (CC126) and DEC-205 (CC98) monoclonal Abs were obtained from Serotec (Raleigh, NC). CD80/86 was detected by human CD152-muIg/R-PE fusion protein (Ancell, Baymont, MN). Isotype specific primary Abs used were anti-mouse IgG1, IgG2b, IgG2a and IgM (Serotec). Secondary Abs (Southern Biotechnology Associates, Birmingham, AL) included anti-IgM PE, anti-IgG2b PE, anti-IgG2b FITC, anti-IgG2a FITC,

and anti-IgM FITC. Anti-IgG1 Tricolor was obtained from Caltag Laboratories (Burlingame, CA). Cells were washed with FACs buffer and resuspended in 2% paraformaldehyde in PBS. Each sample had 10,000 events collected using a Becton Dickinson LSR flow cytometer (San Jose, CA). Analysis of cell surface marker expression was done using FloJo software (Tree Star, Inc., Ashland, OR).

1.9. Tracer endocytosis

FITC conjugated ovalbumin (FITC-OVA; Molecular Probes, Eugene, OR) was used to examine the endocytic abilities of the different DC populations. $CD11c^+$ cell concentrations were adjusted to 1×10^5 cells/ml and incubated at 37 °C or 4 °C for 1 h with FITC-OVA (1 mg/ml). Cells were washed with FACs buffer and resuspended in 2% paraformaldehyde in PBS. 10,000 events were acquired for each sample using a flow cytometer (Becton Dickinson LSR). Data were analyzed using FloJo software (Tree Star, Inc.).

1.10. T cell stimulatory capacity

Allogeneic T cells were labeled with anti-CD4 (17D1; VMRD), magnetic micro beads (Miltenyi Biotech) and positively selected by magnetic activated cell sorting. T cell proliferation was assessed by 5,6-carboxyfluorescein diacetate, succinimidyl ester (CFSE) staining (1 μM/ml; Molecular Probes). CFSE, irreversibly coupled to intracellular proteins is equally divided between daughter cells as a result of cell division. Labeled T cells (2.5×10^5/ml) were resuspended in cRPMI and stimulated with monocyte-derived macrophages, MDDCs or lung DCs at varying stimulator to responder ratios (1:1, 10, 50, 100). Co-cultures were incubated at 37 °C with 5% CO_2 for 3 d. Analysis of CFSE fluorescence intensity of T cells was performed using flow cytometry. Cells were analyzed using ModFit LT™software (Verity Software House Inc., Topsham, ME).

1.11. Statistical analysis

Data were expressed as the mean ± S.E.M. Samples were compared using an unpaired two-tailed Student's *t*-test. Significance was determined by $P < 0.05$.

2. Results

2.1. Localization of $CD11c^+DEC\text{-}205^+$ cells within the lung parenchyma

Previous reports have identified pulmonary DCs within the lung parenchyma of the upper and lower lobes (Stumbles et al., 2003). Frozen lung sections from the upper right lobe of neonatal lamb lungs were stained for the presence of DCs. DAPI was used to stain nuclei to enhance visualization of the overall cellular distribution within the tissue sections. CD11c and DEC-205 staining were observed on cells in alveoli near bronchioles (Fig. 1). The majority of $CD11c^+$ cells co-expressed DEC-205 and these double positive cells were distributed around the alveolar sacs and within the lung parenchyma. Previous reports have identified DEC-205 on subsets of DCs in the mouse, human and bovine species (Gliddon et al., 2004).

2.2. Pulmonary DC morphology

Unique ultrastructural characteristics of lung DCs have been used to positively identify these cells by either confocal or electron microscopy. In the present studies, TEM was utilized to examine the ultrastructure of purified $CD11c^+$ lung DCs. Isolated ovine lung $CD11c^+$ cells exhibited typical DC morphology, including long dendrites and multivacuolar and multilamellar vesicles (Fig. 2A and B). However, these vesicles were less numerous than observed for MDDCs (Fig. 2C).

2.3. Analysis of surface antigen expression

To date, large animal lung DCs have yet to be characterized and our study compares phenotypic data of isolated lung DCs from neonatal and adult sheep. Over ninety percent of all isolated $CD11c^+$ lung DCs co-expressed DEC-205 (Figs. 3A and 4A). Regardless the age, low to moderate expression was observed for DEC-205, CD80/86, MHC class II and CD 14 (Figs. 3B and 4B). The only notable phenotypic difference in lung DCs between neonates and adults was a higher MHC class I expression on neonatal cells (Fig. 3B). When lung DCs were compared to monocyte-derived macrophages, differences were

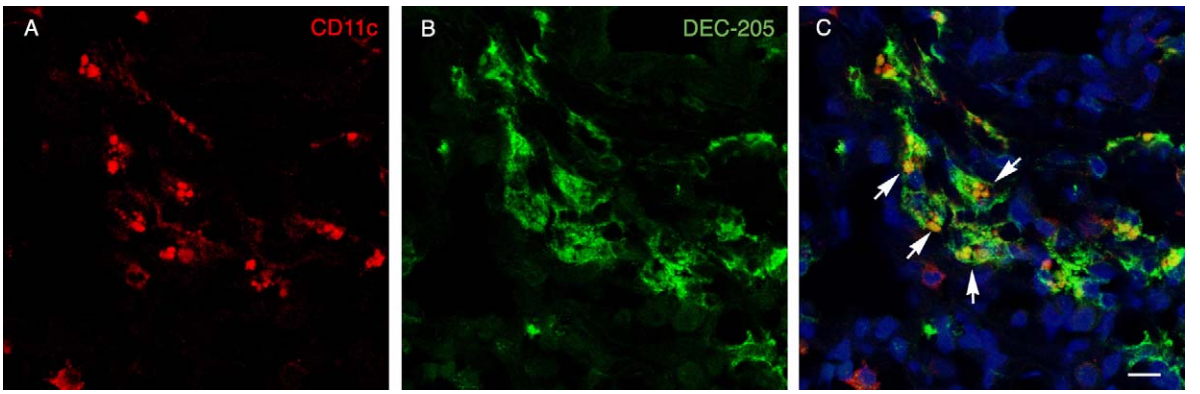

Fig. 1. Immunofluorescence staining of dendritic cells within neonatal ovine lung cryosections. Sections were incubated with primary antibodies directed against CD11c (A) or DEC-205 (B) followed by secondary antibodies fluorescently labeled with Alexa Fluor 633 (red) or Alexa Fluor 488 (green). Multi-color immunofluorescence staining shows dual labeled DCs (C) and nuclear staining with DAPI (blue). Co-expression of molecules is shown by yellow (arrows). Scale bars: A–C, 10 μm.

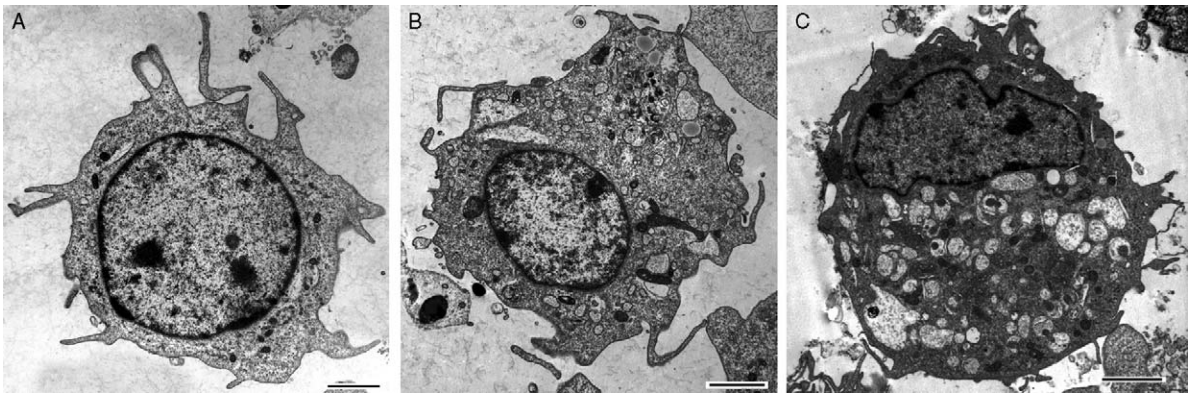

Fig. 2. Transmission electron micrograph (TEM) showing morphological characteristics of sorted $CD11c^+$ lung dendritic cells (A and B) and monocyte-derived dendritic cells (C). Cells were isolated by magnetic activated cell sorting and stained by standard methodology for TEM. Characteristic large diameter mononuclear cells with microvillus projections were observed. Scale bars: A–C, 2 μm.

observed in that macrophages expressed higher CD14 and lower DEC-205, MHC class I, and MHC class II (Fig. 4B). Higher expression of DEC-205 and lower expression of CD14 are both indicative of dendritic cells (Gliddon et al., 2004; Nicod and Cochand, 2001). In both neonates and adults, immature and mature MDDCs demonstrated low expression of all surface molecules when compared to lung DCs except for two molecules in the adults (Tables 1 and 2). Mature, adult MDDCs expressed higher CD80/86 and MHC class I than their lung DCs counterparts (Fig. 4B). $CD11c^+$ DCs were isolated from lymph nodes (LN DCs) of both adult and neonatal sheep for phenotypic comparisons of lung DCs. In general, within age groups the lung and LN DC populations expressed higher levels of most surface antigens when compared to immature MDDCs (Tables 1 and 2).

2.4. Endocytic capabilities

The ability of immature DCs to take up antigen in the lung environment is crucial for generating an immune response to invading pathogens. Several pathways exist for antigen uptake and FITC-OVA was used as a measure of receptor-mediated endocytosis via clathrin-coated pits. There was no observable difference between neonatal and adult lung DCs in their ability to endocytose FITC-OVA (Fig. 5). FITC-

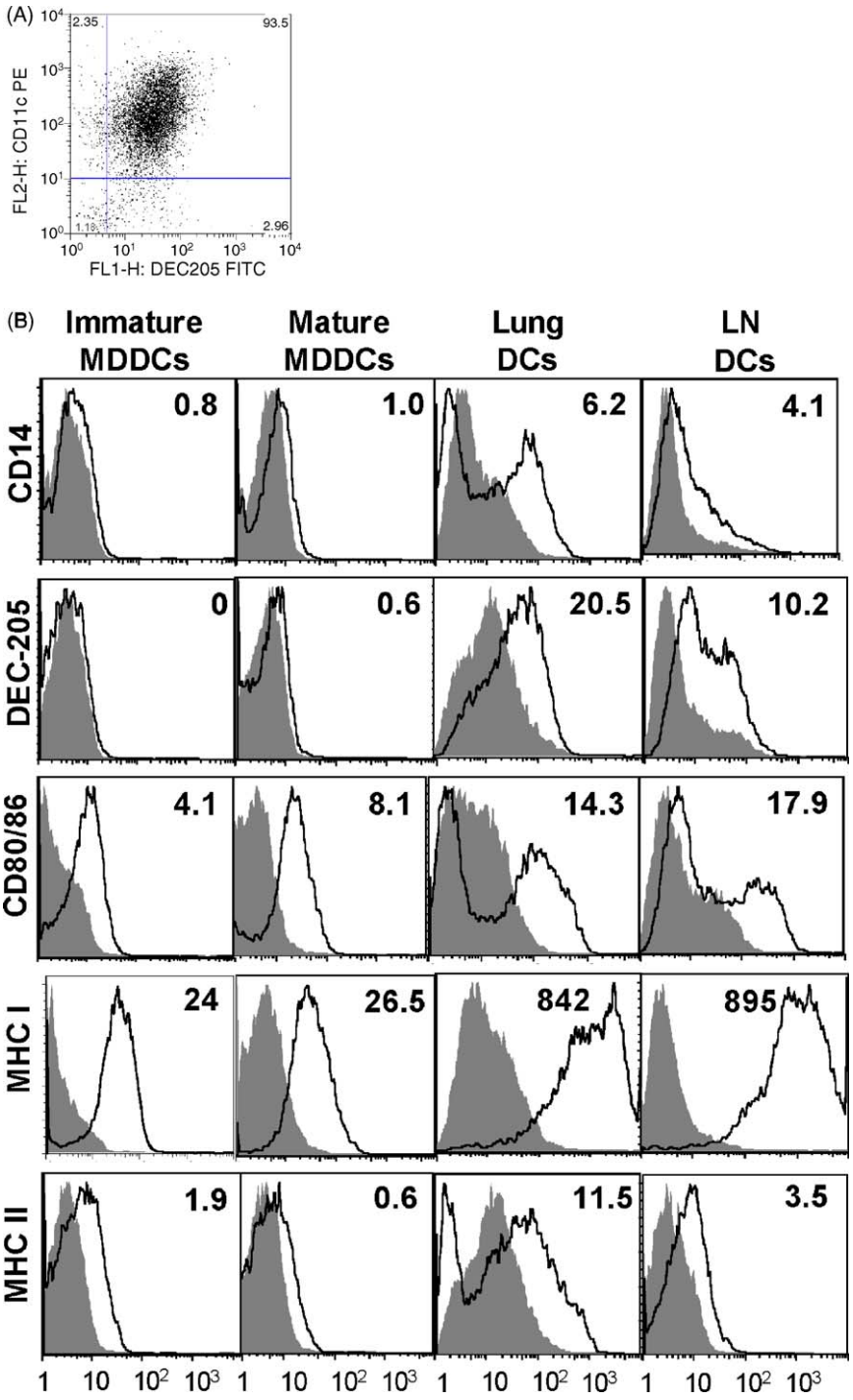

Fig. 3. Flow cytometric analysis of surface antigen expression on neonatal ovine DC populations. Isolated adherent mononuclear cells were cultured for 7 d with supplemented medium containing recombinant human GM-CSF (5 ng/ml) and IL-4 (2 ng/ml). Samples of monocyte-derived dendritic cells (MDDCs; immature) were stimulated with LPS (1 μg/ml) for an additional 3 d to induce maturation. Magnetic sorting

OVA uptake by lung and LN DCs was similar between age groups. Of note was a significantly different ($P < 0.05$) endocytosis observed between the MDDCs and lung DCs. The MDDCs from both age groups were able to endocytose more FITC-OVA than the lung or LN DCs. Consequently, lung DCs isolated in the present experiments cannot be considered fully mature since they are capable of antigen uptake albeit to a lesser degree than immature MDDCs.

2.5. Allogeneic T cell stimulation by isolated lung DCs

One unique characteristic of DCs is their ability to stimulate naive T cells to proliferate. DCs of monocyte, lung and LN origin were used at various concentrations (10^4–10^6) to stimulate allogeneic T cells (2×10^4) labeled with CFSE (Fig. 6). Control monocyte-derived macrophages were included as a comparative population of APCs. As expected, analysis of proliferation as measured by the reduction in CFSE fluorescence revealed weak allogeneic T cell stimulation by macrophages (72% remaining in the parent population). Increasing the ratio of macrophages to responder cells did not increase the T cell proliferative response (data not shown). MDDCs stimulated more allogeneic T cells to proliferate (45% remaining in the parent population) than macrophages ($P < 0.05$). Moreover, lung DCs were also potent stimulators of allogeneic T cell proliferation even at a ratio of 0.5 DC:1 T cell (16% T cells remaining in the parent population). Increasing the ratio of lung DCs to T cells by 5:1 further enhanced allogenic proliferation (4% T cells remaining in the parent population).

3. Discussion

In large animal models, lung dendritic cell biology has not been well characterized. In order to identify lung DCs, a combination of phenotypic characterization, morphological criteria and functional assays were applied. CD11c mAb, BAQ153A, was chosen to sort lung DCs due to its ability to label a majority of afferent lymph dendritic cells and very few lung tissue macrophages in the ovine species (Gupta et al., 1993). Our results demonstrate the presence of $CD11c^+DEC205^+$ cells distributed throughout the lung parenchyma and airway epithelium, which has been confirmed in rodent models and human tissue samples (van Haarst et al., 1994; Schon-Hegrad et al., 1991; Gong et al., 1992; Takano et al., 2005). DEC-205 (CD205) is a C-type lectin that functions as an endocytic receptor on DCs and is present on both immature and mature DC populations (Gliddon et al., 2004). Expression of DEC-205 is higher on DCs compared to macrophages or lymphocytes. Phenotypic analysis showed that the isolated cell-surface characteristics were generally lower CD14 and higher DEC-205, CD80/86, MHC class II as compared to monocyte-derived macrophages. Moreover, the lung derived cells were effective stimulators in allogenic MLRs, a hallmark feature of DCs.

Enzymatic digestion and magnetic activated cell sorting of ovine lung tissue resulted in a population of DCs that exhibited characteristic morphology of dendrites, multivacuolar cytoplasm and typical size for DCs. Morphological differences between MDDCs and lung DCs were noted with the former having more vacuoles present in the cytoplasm. Processing of DCs may alter their phenotype and functional properties, but our data is consistent with lung DCs isolated from rodents and human lung samples (Schon-Hegrad et al., 1991; Calder et al., 2004; Cochand et al., 1999; Gonzalez-Juarrero et al., 1999). In general, ovine lung DCs are phenotypically similar to their rodent counterparts and express comparable low levels of CD14, CD80/86 and moderate levels of MHC class II (Calder et al., 2004). Moreover, studies from human lung DCs reported low expression of CD14, CD80 and CD86 but high MHC class II expression (Cochand et al., 1999). Thus, as compared to MDDCs, it is evident that lung DCs can be considered phenotypically between immature and mature stages. Fine phenotypic discrimination of ovine lung DC subsets awaits further study.

was used to purify $CD11c^+$ cells from digested suspensions of neonatal lungs (Lung DCs) or tracheobronchial and mediastinal lymph nodes (LN DCs). Over ninety percent of the neonatal lung DCs (A) co-expressed CD11c and DEC-205. For the cell surface antigens examined, antigens on neonatal lung DCs exceeded that of cell surface antigens expressed on MDDCs (B). Shaded histograms represent isotype control antibodies. Number shown in the upper right corner of each plot is the mean fluorescence intensity (MFI) for the cell surface antigen minus the MFI of the isotype control. Data shown are representative of results obtained from 10 neonatal lambs.

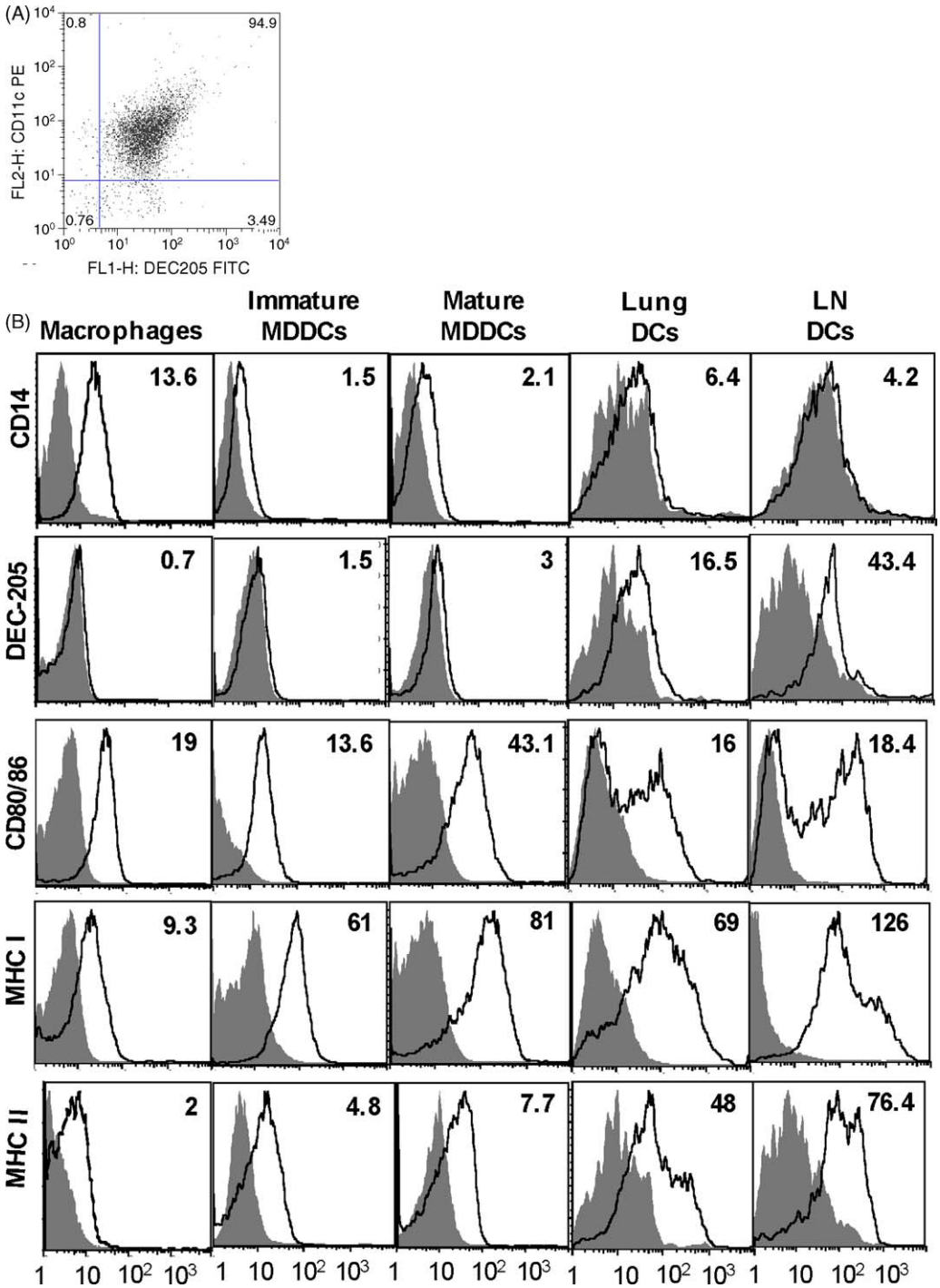

Fig. 4. Flow cytometric analysis of surface antigen expression on adult ovine macrophage or DC subsets. Isolated adherent mononuclear cells were cultured for 7 d with supplemented medium to obtain monocyte-derived macrophages. DC populations were derived as described in Fig. 3. The majority of adult lung DCs (A) were CD11c and DEC-205 double positive. Adult lung DCs (B) expressed higher levels of DEC-205, MHC

Table 1
Expression of selected cell-surface molecules on neonatal ovine dendritic cell populations

	CD14	DEC-205	CD80/86	MHC I	MHC II
Immature MDDCs	0.9 ± 0.1	0 ± 0	4.8 ± 1.1	22.3 ± 3.6	1.9 ± 0.1
Mature MDDCs	0.9 ± 0.1	0.4 ± 0.2	7.4 ± 1.0	25.8 ± 5.5	0.6 ± 0
Lung DCs	$6.1 \pm 1.5^{a,b}$	$20.6 \pm 5.7^{a,b}$	27.2 ± 17.1	$833.3 \pm 267.3^{a,b}$	18.6 ± 7.1
LN DCs	4.5 ± 1.2^{c}	10.3 ± 0.9^{c}	15.0 ± 2.9^{c}	991.1 ± 131.1^{c}	4.0 ± 1.7

Data are expressed in terms of mean fluorescence intensity (MFI) of the cell surface antigen minus the MFI of the appropriate isotype control ± S.E.M. The data set includes 10 neonatal lambs.

[a] Significantly higher compared to immature MDDCs ($P < 0.05$).

[b] Significantly higher compared to mature MDDCs ($P < 0.05$).

[c] Significantly higher compared to immature MDDCs ($P < 0.05$).

Table 2
Expression of selected cell-surface molecules on adult ovine macrophage and dendritic cell populations

	CD14	DEC-205	CD80/86	MHC I	MHC II
Macrophages	13.2 ± 0.6	0.9 ± 0.2	16.2 ± 3.8	7.0 ± 2.4	2.2 ± 0.2
Immature MDDCs	2.1 ± 0.2	1.2 ± 0.3	19.5 ± 6.4	61.9 ± 4.6	5.5 ± 0.7
Mature MDDCs	2.2 ± 0.4	2.5 ± 0.5	38.4 ± 8.6	123.1 ± 44.2	6.7 ± 1.4
Lung DCs	$8.9 \pm 4.4^{a,b}$	$13.5 \pm 3^{b,c}$	14.4 ± 3.7^{b}	61.7 ± 11^{c}	36.3 ± 13.6
LN DCs	5.7 ± 2.6	37.7 ± 10.3	17.4 ± 4.3	128.9 ± 22.1^{d}	62.1 ± 16.1^{d}

Mean fluorescence intensity (MFI) of the cell surface antigen minus the MFI of the appropriate isotype control ± S.E.M. is shown. The data set includes four adult sheep.

[a] Significantly higher compared to immature MDDCs ($P < 0.05$).

[b] Significantly higher compared to mature MDDCs ($P < 0.05$).

[c] Significantly higher compared to monocyte-derived macrophages ($P < 0.05$).

[d] Significantly higher compared to immature MDDCs ($P < 0.05$).

Adult and neonatal rodent and human lung samples have been utilized for the characterization of pulmonary DCs (Schon-Hegrad et al., 1991; McCarthy et al., 1992; Calder et al., 2004; Demedts et al., 2005). To date, characterization of neonatal lung DCs in a large animal species have not yet been reported. We were interested in addressing the question of whether or not phenotypic or functional differences existed between adult and neonatal lung DCs due to the ongoing debate concerning the maturity of the immune response in neonates (Adkins et al., 2004; Goriely et al., 2001; Muthukkumar et al., 2000). Recently, isolated $CD11c^{+}$ DCs from the spleens of neonatal mice were shown to be functionally competent in stimulating Th1 responses in the adult murine microenvironment (Sun et al., 2005). Their data suggest that MHC class II and CD86 expression is similar on both neonatal and adult splenic DCs. Our observations support their findings in that lung and LN DCs from both neonates and adults have similar expression levels of MHC class II, CD80/86 and CD14. One notable difference between the age groups was the markedly higher expression of MHC class I on neonatal lung DCs. At present, it is not clear why neonatal lung DCs express such high levels of MHC class I. Overall, neonatal and adult ovine lung DCs exhibit similar phenotypic characteristics.

Numerous studies have examined in vitro-derived DCs (e.g., bone marrow or monocyte-derived), primarily due to the relative ease with which sufficient numbers of cells can be obtained. In the present

class I and MHC class II, but lower levels of CD14 compared to monocyte-derived macrophages. Shaded histograms represent staining with isotype-matched control antibodies. Number shown in each panel is the mean fluorescence intensity (MFI) for staining of the indicated cell surface antigen minus the MFI of staining with the isotype control antibody. Data shown are representative of results obtained from four adult sheep.

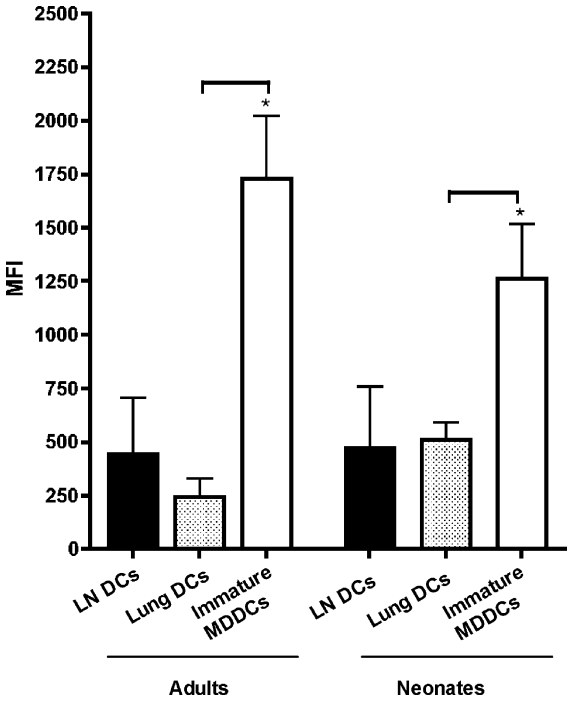

Fig. 5. Uptake of FITC-ovalbumin (FITC-OVA) by various DC populations. Lung, LN and monocyte-derived DCs were adjusted to a concentration of 1×10^5 cells/ml and incubated with FITC-OVA for 1 h at 37 °C or 4 °C. Delta mean fluorescence intensities (MFI) shown are the values at 37 °C minus the values at 4 °C. Statistical analyses indicated that uptake of FITC-OVA by monocyte-derived DCs was significantly greater than that observed for lung or LN DCs ($^*P < 0.05$). Uptake of FITC-OVA did not differ between neonatal and adult DCs.

experiments, MDDCs were used as a basis for comparison. In contrast to lung DCs, phenotypic expression of cell surface antigens on MDDCs differed between neonates and adults. Adult sheep MDDCs expressed higher levels of the co-stimulatory molecule CD80/86, MHC class I and MHC class II than the neonatal MDDCs. Levels of expressions for these surface antigens, as well as CD14 are in agreement with a previous report characterizing MDDCs from adult sheep (Chan et al., 2002). Based on our research and that of others (Calder et al., 2004; Cochand et al., 1999; Gonzalez-Juarrero and Orme, 2001), it is apparent that tissue derived DCs differ from their in vitro-derived counterparts. Whether the parameters that define immature and mature in vitro-derived DCs are adequate for distinguishing maturation stages of tissue-derived DCs is not clear.

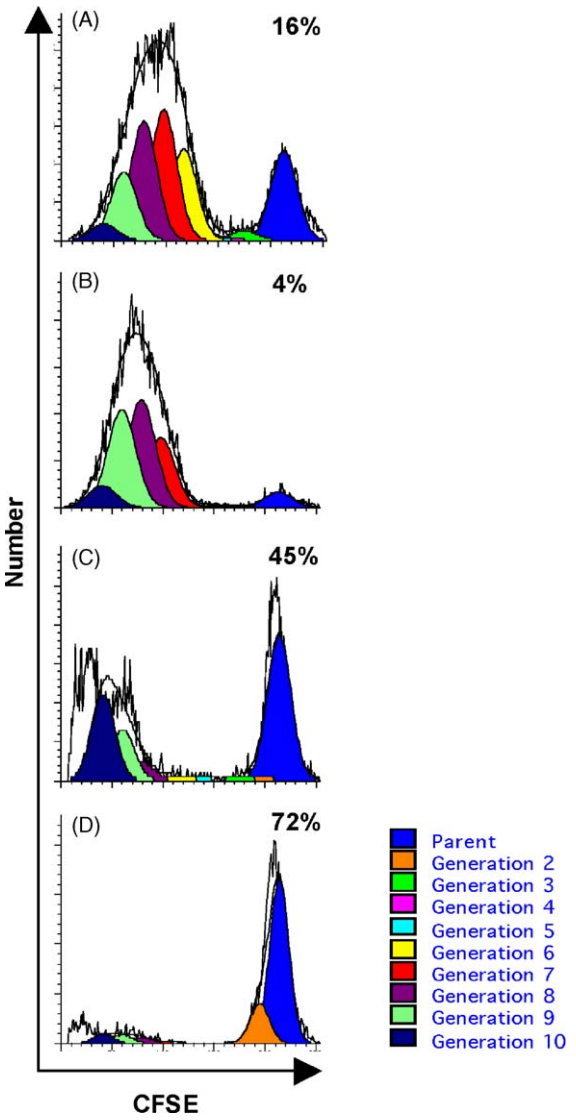

Fig. 6. Allogeneic T cell stimulation by macrophages or DC populations. Isolated T cells were labeled with CFSE and co-cultured with neonate lung DCs (A and B), monocyte-derived DCs (C) or monocyte-derived macrophages (D) for 3 d. T cell proliferation was assessed by CFSE staining which irreversibly couples to intracellular proteins and is equally divided among daughter cells as a result of cell proliferation. ModFit LT analysis reveals the T cell parent population and daughter generations following stimulation with the various APC populations (A0.5:1,B5:1,C1:1,D1:1 ratio of stimulator to responder cells). The number presented in the right upper hand corner of each panel is the percentage of cells remaining in the parent T cell population. Lung DCs were potent stimulators of allogeneic T cell proliferation as compared to monocyte-derived macrophages. Data shown are representative of four neonatal lambs.

Immature DCs are known to take up antigen via receptor-mediated endocytosis and macropinocytosis. As a functional measure of maturation state, ovine lung DCs from both neonates and adults were incubated with FITC-OVA to measure their endocytic capabilities. The age of the animal had no significant effect on the lung DCs' ability to take up FITC-OVA. Statistically significant differences in the ability to endocytose the fluorescent-labeled tracer molecules were exhibited between lung DC and MDDC populations. Lung DCs were functionally more mature than MDDCs as evidenced by lower uptake via clathrin-coated pits. These results parallel previous studies that have reported reduced uptake of a tracer antigen by lung DCs when compared to immature MDDCs (Cochand et al., 1999). A hallmark of a fully mature DC is their inability to uptake antigen. Based on this criterion, lung DCs have not attained full maturation, but rather exist in a semi-mature state.

The ability to stimulate naïve T cells to proliferate is a defining characteristic of DCs. The mixed leukocyte reaction has been used as a measure of responder T cell proliferation against antigens presented by allogeneic stimulator DCs. Utilizing CFSE labeling of allogenic T cells has been demonstrated as an effective way to quantify MLR assays (Chen et al., 2003). As expected, ovine lung DCs exhibited this distinguishing feature of potent stimulators of T cell proliferation when compared to monocyte-derived macrophages. In fact, isolated lung DCs were the most potent of the APCs examined at stimulating allogeneic T cells to proliferate as measured by the percentage of daughter T cells generated during 5 d of co-culture. In contrast, monocyte-derived macrophages stimulated few allogeneic T cells to proliferate with the majority of these cells remaining in the parent population. These data are similar to other rodent and human studies that report lung DCs exhibiting strong abilities to stimulate allogeneic and antigen specific T proliferation (Calder et al., 2004; Cochand et al., 1999; Demedts et al., 2005). In summary, isolated ovine lung DCs are functionally able to stimulate allogeneic T cell proliferation.

Neonates generate nonprotective immune responses to specific respiratory pathogens resulting in increased morbidity and mortality when compared to adults. The similarities described here between neonate and adult lung DCs suggest the immaturity of neonatal DCs cannot be the reason for the ineffective immune response. It is plausible to suggest that the neonatal lung microenvironment is critical to the development of an immune response that does not protect. The results of the present studies have important implications for the design of effective vaccines for use against respiratory pathogens in neonates.

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